# Center for Veterinary Biologics
# and
# National Veterinary Services Laboratories
# Testing Protocol

## Supplemental Assay Method for the Titration of Porcine Rotavirus Antibody
### (Constant Virus-Varying Serum Method)

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**Supplemental Assay Method for the Titration of Porcine Rotavirus Antibody (Constant Virus-Varying Serum Method)**

# 1. Introduction

## 1.1 Background

This Supplemental Assay Method (SAM) is an *in vitro* serum neutralization (SN) method which utilizes cytopathic effects (CPE) or an indirect fluorescent antibody (IFA) technique in a cell culture system for determining the SN antibody titer against Group A porcine rotavirus (PROTA). The SN assay uses a constant amount of virus to test varying dilutions of serum.

**Note: For this SAM, the dilution terminology of 1:10, 1:20, etc., specifies 1 part plus 9 parts (liquid), 1 part plus 19 parts, etc.**

## 1.2 Keywords

Porcine rotavirus, PROTA, CPE, IFA, SN, titer, *in vitro*

# 2. Materials

## 2.1 Equipment/instrumentation

**2.1.1** Incubator,[1] $36^0 \pm 2^0$C, 5% ± 1% $CO_2$, high humidity

**2.1.2** Water bath[2]

**2.1.3** Microscope,[3] inverted light

**2.1.4** Microscope,[4] ultraviolet (UV) light

**2.1.5** Vortex mixer[5]

**2.1.6** Micropipetters: 200 µl and 1000 µl single channel;[6] 300 µl x 12 channel[7]

[1] Model 3158, Forma Scientific, Inc., Box 649, Marietta, OH 45750-0649 or equivalent
[2] Cat. No. 15-461-10, Fisher Scientific, Inc., 319 West Ontario, Chicago, IL 60610 or equivalent
[3] Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747-3157 or equivalent
[4] Model BH2, Olympus America, Inc. or equivalent
[5] Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 70 Orville Dr., Bohemia, NY 11716 or equivalent
[6] Pipetman®, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent
[7] Finnpipettes®, Cat. No. NX204662D, A. Daigger Co., Inc., 199 Carpenter Ave., Wheeling, IL 60090 or equivalent

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## 2.2 Reagents/supplies

**2.2.1** PROTA Reference Viruses[8]

**2.2.1.1** Serotype 4 (Gottfried strain)

**2.2.1.2** Serotype 5 (OSU strain)

**2.2.2** Rhesus monkey kidney cells (MA-104)[9] free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR)

**2.2.3** Minimum essential medium (MEM)

**2.2.3.1** 9.61 g MEM[10]

**2.2.3.2** 2.2 g sodium bicarbonate ($NaHCO_3$)[11]

**2.2.3.3** Q.S. to 1000 ml with deionized water (DW); adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[12]

**2.2.3.4** Sterilize through a 0.22-µm filter.[13]

**2.2.3.5** Aseptically add:

**1.** 10 ml L-glutamine[14]

**2.** 2.5 µg/ml amphotericin B[15]

**3.** 100 units/ml penicillin[16]

**4.** 50 µg/ml gentamicin sulfate[17]

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[8] Reference quantities are available on request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent
[9] Available on request from the CVB-L or equivalent
[10] MEM with Earle's salts without sodium bicarbonate, Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent
[11] Cat. No. S 5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent
[12] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent
[13] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent
[14] L-glutamine-200 mM (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent
[15] Cat. No. A 2411, Sigma Chemical Co. or equivalent
[16] Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent.
[17] Cat. No. 0061-0464-04, Schering Laboratories or equivalent

**5.** 100 µg/ml streptomycin[18]

**2.2.3.6** Store at 4⁰ ± 2⁰C.

**2.2.4** Growth Medium

**2.2.4.1** 930 ml of MEM

**2.2.4.2** Aseptically add 70 ml of heat-inactivated fetal bovine serum (FBS).

**2.2.4.3** Store at 4⁰ ± 2⁰C.

**2.2.5** Diluent Medium

**2.2.5.1** 100 ml MEM

**2.2.5.2** 83.3 µl pancreatin[19]

**2.2.5.3** Store at 4⁰ ± 2⁰C.

**2.2.6** Anti-PROTA monoclonal antibody (MAb)[9]

**2.2.6.1** MAb against Serotype 4 (Gottfried strain)

**2.2.6.2** MAb against Serotype 5 (OSU strain)

**2.2.7** Rabbit anti-mouse fluorescein isothiocyanate labeled conjugate[20] (Rabbit Anti-mouse Conjugate)

**2.2.8** 0.01 M Phosphate buffered saline (PBS)

**2.2.8.1** 1.19 g sodium phosphate, dibasic, anhydrous ($Na_2HPO_4$)[21]

**2.2.8.2** 0.22 g sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)[22]

**2.2.8.3** 8.5 g sodium chloride (NaCl)[23]

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[18] Cat. No. S 9137, Sigma Chemical Co. or equivalent
[19] Pancreatin 4XNF (10X), Cat. No. 610-5720AG, Life Technologies, Inc. or equivalent
[20] Cat. No. 04-6111, Zymed Laboratories, Inc., 458 Carlton Ct., So. San Francisco, CA 94080 or equivalent
[21] Cat. No. S 0876, Sigma Chemical Co. or equivalent
[22] Cat. No. S 9638, Sigma Chemical Co. or equivalent
[23] Cat. No. S 9625, Sigma Chemical Co. or equivalent

**2.2.8.4** Q.S. to 1000 ml with DW.

**2.2.8.5** Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH)[24] or 2.0 N HCl.

**2.2.8.6** Sterilize by autoclaving at 15 psi, 121$^0$ ± 2$^0$C for 35 ± 5 min.

**2.2.8.7** Store at 4$^0$ ± 2$^0$C.

**2.2.9** 80% Acetone

**2.2.9.1** 80 ml acetone[25]

**2.2.9.2** 20 ml DW

**2.2.9.3** Store at room temperature (RT) (23$^0$ ± 2$^0$C).

**2.2.10** Cell culture plates,[26] 96 well

**2.2.11** Polystyrene tubes,[27] 12 x 75 mm

## 3. Preparation for the test

### 3.1 Personnel qualifications/training

Personnel must have training in the immunological basis of SN assays, cell culture techniques, the principles of IFA, and aseptic technique.

### 3.2 Preparation of equipment/instrumentation

**3.2.1** On the day of test initiation, set a water bath at 56$^0$ ± 2$^0$C.

**3.2.2** On the day of test initiation, set a water bath at 36$^0$ ± 2$^0$C.

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[24] Cat. No. 925-30, Sigma Chemical Co. or equivalent
[25] Cat. No. A 6015, Sigma Chemical Co. or equivalent
[26] Costar® 3596, Costar Corp., 1 Alewife Center, Cambridge, MA 02140 or equivalent
[27] Falcon® 2058, Becton Dickinson Labware, 2 Bridgewater Ln., Lincoln Park, NJ 07035 or equivalent

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## 3.3 Preparation of reagents/control procedures

**3.3.1** MA-104 Plates. Two days prior to test initiation, seed 96-well cell culture plates with MA-104 cells, in Growth Medium, at a cell count that will produce a monolayer after 48 ± 8 hr of incubation at 36⁰ ± 2⁰C in a $CO_2$ incubator. These become the MA-104 Plates. Two sera can be tested on each MA-104 Plate. Growth Medium is changed if excess acidity of the medium is observed or cells are not confluent after incubation.

**3.3.2** Stock Virus Preparation. On the day of test initiation, rapidly thaw vials of each PROTA Reference Virus in a 36⁰ ± 2⁰C water bath. Dilute each virus in Diluent Medium to contain 100-700 50% tissue culture infective dose ($TCID_{50}$)/200 µl.

**3.3.3** Virus Back Titration. On the day of test initiation, make 4 serial tenfold dilutions of each Stock Virus.

**3.3.3.1** Place 900 µl of MEM into 2 sets of 8, 12 x 75-mm polystyrene tubes, labeled $10^{-1}$ to $10^{-4}$. Label each set with the appropriate stock virus.

**3.3.3.2** Transfer 100 µl of each Stock Virus into the appropriate $10^{-1}$ tubes; mix by vortexing. Discard pipette tip.

**3.3.3.3** Transfer 100 µl from the $10^{-1}$ tube to the $10^{-2}$ tube; mix by vortexing. Discard pipette tip.

**3.3.3.4** Repeat **Section 3.3.3.3** for the remaining tubes, transferring 100 µl sequentially from the previous dilution to the next dilution until the dilution sequence is completed. Discard 100 µl from each $10^{-4}$ tube.

**3.3.3.5** Add 900 µl of Diluent Medium to each set of tubes. This becomes the Virus Back Titration-Diluent Medium Mixture.

**3.3.4** Working Anti-PROTA MAb. On the day of MA-104 plate examination, if an IFA test is to be conducted, dilute the appropriate Anti-PROTA MAb in PBS, according to the CVB-L supplied Reference and Reagent Sheet or as determined for that specific MAb.

**3.3.5** Working Rabbit Anti-mouse Conjugate. On the day of MA-104 plate examination, if an IFA test is to be conducted, dilute the Rabbit Anti-mouse Conjugate in PBS, according to the manufacturer's recommendations.

## 3.4 Preparation of the sample

**3.4.1** On the day of test initiation, heat inactivate all Test Sera in a $56^0 \pm 2^0$C water bath for 30 ± 5 min.

**3.4.2** Prepare serial twofold dilutions of Test Sera in 96-well cell culture plates, which become the Dilution Plates (**see Appendix I**). Place each Test Serum onto 2, 96-well cell culture plates, 1 for each PROTA serotype to be tested. Make twofold dilutions as follows:

**3.4.2.1** Add 150 µl Diluent Medium to all wells in Rows B-H.

**3.4.2.2** Add 150 µl of a Test Serum to Rows A and B. Change pipette tips. Mix Row B with a multichannel micropipettor (6-8 fills).

**3.4.2.3** Transfer 150 µl from Row B to Row C. Change pipette tips. Mix Row C with a multichannel micropipettor (6-8 fills).

**3.4.2.4** Continue as in **Section 3.4.2.3** for the remaining rows. Discard 150 µl from all wells in Row H.

**3.4.2.5** Add 150 µl of Stock Virus to all wells of the Dilution Plate. Tap plates gently to mix.

**3.4.2.6** Incubate for 60 ± 10 min at 36⁰ ± 2⁰C to allow for neutralization of virus. This is an additional twofold dilution of the Test Sera. This becomes the Virus-Test Sera Mixture.

## 4. Performance of the test

**4.1** On the day of test initiation, decant Growth Medium from the MA-104 Plates.

**4.2** Add 200 µl/well Diluent Medium to the MA-104 Plates. Decant the Diluent Medium.

**4.3** Again, add 200 µl/well Diluent Medium to the MA-104 Plates. Incubate for 60 ± 10 min at 36⁰ ± 2⁰C. Decant the Diluent Medium.

**4.4** Inoculate 200 µl/well of each Virus-Test Sera Mixture into 5 wells/dilution of an MA-104 Plate, using a multichannel pipettor.

**4.5** Inoculate 200 µl/well of each dilution ($10^{0}$ to $10^{-4}$) of Virus Back Titration-Diluent Medium mixture into 5 wells of an MA-104 Plate, using a multichannel pipettor.

**4.6** Add 200 µl/well of Diluent Medium to 2 columns on each MA-104 Plate, to serve as uninoculated cell controls.

**4.7** Incubate the MA-104 Plates for 120 ± 12 hr postinoculation (HPI) at 36⁰ ± 2⁰C.

**4.8** CPE counting is the primary method of determining the $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$).

**4.8.1** 120 ± 12 HPI, examine the wells with an inverted light microscope. The CPE of PROTA is visible as cell death in the cell monolayer.

**4.8.2** Record the number of wells/dilution showing any characteristic CPE of PROTA for each Test Serum and Virus Back Titration.

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**4.8.3** Calculate the $TCID_{50}$ of each Virus Back Titration using the Spearman-Kärber method as commonly modified.

**4.8.4** Calculate each endpoint of the Test Sera using the Spearman-Kärber method as commonly modified. The endpoints of the Test Sera are reported as SN titer which corresponds to the reciprocal of the highest serum dilution that neutralizes PROTA.

Example:

1:2 dilution of Test Sera = 5 of 5 wells CPE
1:4 dilution of Test Sera = 5 of 5 wells CPE
1:8 dilution of Test Sera = 3 of 5 wells CPE
1:16 dilution of Test Sera = 0 of 5 wells CPE

Titer = (X - d/2 + [d * S]) where:

X = $Log_{10}$ of lowest dilution (=0.3)
d = $Log_{10}$ of dilution factor (=0.3)
S = Sum of proportion of CPE - (13/5=2.6)
Titer = (0.3 - 0.3/2 + [0.3 * 13/5]) = 0.93
antilog of 0.93 = 8.5

Titer of the Test Serum is 1:9

**4.9** Certain strains of PROTA may not exhibit pronounced CPE, thus an IFA may be conducted to determine the titer:

**4.9.1** Decant the Growth Media from the MA-104 Plates.

**4.9.2** Rinse the MA-104 Plates with PBS; incubate at RT for 5 ± 2 min. Decant the PBS.

**4.9.3** Fill wells with 80% Acetone.

**4.9.4** Incubate at RT for 15 ± 5 min.

**4.9.5** Decant the 80% Acetone from the MA-104 Plates and air dry at RT.

**4.9.6** Pipette 35 µl of the Working Anti-PROTA MAb into all wells. Incubate for 45 ± 15 min at RT.

**4.9.7** Fill the wells completely with PBS; incubate at RT for 5 ± 2 min. Decant the PBS.

**4.9.8** Repeat for a total of 2 washes.

**4.9.9** Gently tap the MA-104 Plates onto paper towels to remove excess moisture.

**4.9.10** Pipette 35 µl of the Working Rabbit Anti-mouse Conjugate into all wells. Incubate for 40 ± 10 min at RT.

**4.9.11** Repeat **Sections 4.9.7 through 4.9.9.**

**4.9.12** Dip the plate in DW; decant. Allow to air dry or dry at $36^0$ ± $2^0$C.

**4.9.13** Examine the MA-104 Plates with a UV-light microscope at 100 to 200 X magnification.

**4.9.14** A well is considered positive if typical cytoplasmic, apple-green fluorescence is observed.

**4.9.15** Record and calculate as in **Sections 4.8.2 through 4.8.4**

## 5. Interpretation of the test results

**5.1** The test is invalid if CPE, fluorescence, or bacterial/fungal contamination is observed in any of the control wells.

**5.2** For a valid assay, the Virus Back Titration must be between 50 and 350 $TCID_{50}$/200 µl.

## 6. Report of test results

Record all test results on the test record.

## 7. References

**7.1** Code of Federal Regulations, Title 9, Part 113.200, U.S. Government Printing Office, Washington, DC, 2000.

**7.2** Conrath TB. Handbook of Microtiter Procedures. In: *Clinical and Research Applications Laboratory*. Alexandria, VA: Cooke Engineering Co, 1972.

**7.3** Finney DJ. *Statistical Method in Biological Assay*. 3rd ed. London: Charles Griffin and Co, 1978.

**7.4** Rose NR, Friedman H, and Fahey JL, eds. Neutralization Assays. In: *Manual of Clinical Laboratory Immunology*. Washington, DC, ASM, 1986.

**7.5** Parker RA, Pallansch MA. Using the virus challenge dose in the analysis of virus neutralization assays. Statistics in Medicine 11:1253-1262, 1992.

## 8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.

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## 9. Appendix

### Transfer Plate

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** 1:2 | TS1 | TS1 | TS1 | TS1 | TS1 | CC | CC | TS2 | TS2 | TS2 | TS2 | TS2 |
| **B** 1:4 | | | | | | | | | | | | |
| **C** 1:8 | | | | | | | | | | | | |
| **D** 1:16 | | | | | | | | | | | | |
| **E** 1:32 | | | | | | | | | | | | |
| **F** 1:64 | | | | | | | | | | | | |
| **G** 1:128 | | | | | | | | | | | | |
| **H** 1:256 | | | | | | | | | | | | |

TS= Test Serum
CC= Cell Control